Shaping the chromatic characteristics of red wines by using biofilm-detached cells of Starmerella bacillaris strains

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2 ***Starmerella bacillaris* strains**

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Abstract

The aim of this study was to evaluate the effects of 10 *Starmerella bacillaris* strains inoculated as planktonic or biofilm-detached cells on the chromatic characteristics of Montepulciano d'Abruzzo wine. Wines inoculated with biofilm-detached cells of *St. bacillaris* were characterized by a higher content of glycerol and viable yeast cells and a lower content of ethanol than those obtained with planktonic cells. Pyruvic acid content ranged from 45.99 mg/L to 48.19 mg/L and from 41.13 mg/L to 45.9 mg/L in wines fermented with biofilm-detached and planktonic cells, respectively. Wines obtained with biofilm-detached cells showed levels of anthocyanins ranging from 506.8 mg/L to 659.9 mg/L, while those fermented with free cells of *St. bacillaris* ranged from 518 mg/L to 612.6 mg/L. Similarly, the content of polyphenols was higher in wines inoculated with biofilm-detached cells. The different amounts of these compounds resulted in differences in the wine's color. Wines obtained with biofilm-detached cells of *St. bacillaris* had lower b* and h* values than those obtained with planktonic cells. These wines also showed higher a* values, indicating the presence of a stronger red color than the others, and lower clarity (L*). Moreover, the data obtained highlighted that it is possible to predict the color of young wines from must measurements. Further studies will be done to evaluate the role of other non-Saccharomyces yeasts, grown under different aggregation states, in the definition of wine color.

Keywords: *Starmerella bacillaris*; biofilm-detached cells; planktonic cells; anthocyanins; wine color; Montepulciano d'Abruzzo

1. Introduction

The process of wine production encompasses a broad range of microorganisms with distinct functions (Jolly, Varela, & Pretorius, 2014). Yeasts and lactic acid bacteria (LAB) are the main components of the wine microbial consortium, and are known to exhibit either positive or negative effects (Jolly et al., 2014). Although *Saccharomyces cerevisiae* is commonly the dominant species, it is widely acknowledged that a diverse range of non-*Saccharomyces* yeasts are also present in both spontaneous and inoculated wine fermentations. Non-*Saccharomyces* yeasts play a significant role in the release of secondary metabolites contributing to the development of wine's flavor profile (Padilla, Gil, & Manzanares, 2016). In fact, these yeasts are involved in the production of esters, higher alcohols, acids and terpenes (for a review see Padilla et al., 2016). These compounds are essential in the definition of wine organoleptic properties and play an important role in consumer preference (Madžgalj et al., 2022).

During the fermentation process a metabolic interplay between *S. cerevisiae* and non-*Saccharomyces* yeast species has been described, indicating that they do not merely coexist in a passive manner (Jolly et al., 2014). For instance, mixed fermentation of *S. cerevisiae* and *Starmerella bacillaris* (also known as *Candida zemplinina*) allow to enhance fermentation kinetics while minimizing the production of ethyl acetate and acetic acid (Tofalo et al., 2016). This non-*Saccharomyces* yeast is commonly isolated from grapes, musts, soil, fruits, and insects, and exhibits noteworthy oenological traits e.g., elevated glycerol production, reduction of acetic acid and ethanol concentration, enhanced aroma complexity, capacity to thrive in high sugar concentrations, and fructophilic tendencies (Tofalo et al., 2012; Russo et al., 2020; Nadai, Giacomini, & Corich, 2021; Nisiotou et al., 2018). Moreover, the inoculation of *St. bacillaris* adhered on oak chips allowed to improve the color of Trebbiano Abruzzese wines improving its green/yellow nuances (Perpetuini et al., 2023). The impact of yeasts on wine color can be attributed to three distinct mechanisms. Firstly, yeasts can release metabolites that can contribute to the stabilization of red wine color and enhance the content of stable pigments (Escott, Feuillat, Dulau, & Charpentier, 2018). Secondly, yeasts possess enzymatic activities such as

78 glycosidase and pectinase which favor polyphenols extraction form grapes. Finally, yeast cell walls
79 have the ability to adsorb phenolic compounds, particularly anthocyanins and tannins, resulting in a
80 significant reduction in red wine color and astringency (Tofalo, Suzzi, & Perpetuini, 2021). This
81 phenomenon is strain dependent and not yet completely understood. It probably depends on cell wall
82 surface structure and composition being apolar anthocyanins better adsorbed than polar ones.
83 Moreover, the impact of yeasts on wine color is also related to the production of acetaldehyde and
84 pyruvic acid (Morata et al., 2012; Belda et al., 2017). This activity is strain dependent and is notably
85 pronounced in non-*Saccharomyces* yeasts. It has been observed that *Schizosaccharomyces pombe*
86 released a greater concentration of pyruvate compared to *Saccharomyces cerevisiae*. In comparison
87 to *S. cerevisiae*, *Torulaspora delbrueckii* is known to generate a reduced quantity of acetaldehyde.
88 Therefore, the identification of non-*Saccharomyces* yeast strains exhibiting optimal pyruvate and
89 acetaldehyde production for co-fermentation with *S. cerevisiae* could serve as a viable approach to
90 stabilize wine color (Morata et al., 2012; Belda et al., 2017). It seems that the augmentation of
91 metabolic activity and survival time of non-*Saccharomyces* yeasts can lead to a successful mixed
92 culture fermentation also in terms of wine color.
93 Recently, there has been a growing interest in the biofilm lifestyle of yeast and bacteria. Biofilms can
94 be defined as a community of microorganisms that are enclosed by an extracellular matrix composed
95 of extracellular polymeric substances that are generated by the microorganisms themselves (Donlan
96 & Costerton, 2002). Recent studies have reported that biofilm-detached cells are characterized by
97 phenotypes and properties similar to sessile cells and different from those of planktonic ones
98 (Perpetuini et al., 2021, Perpetuini, Tittarelli, Perla, & Tofalo, 2022; Bastard et al., 2016). Therefore,
99 some authors suggested the use of sessile cells, as well as biofilm-detached cells, to shape the
100 oenological parameters of red and white wines (Perpetuini et al., 2021; 2023; Bastard et al., 2016;
101 Pannella et al., 2020). However, to date, little information is available on the influence of biofilm
102 detached yeasts on the chromatic characteristics of wine. Therefore, the aim of this study was to

evaluate the impact of biofilm-detached and planktonic cells of *St. bacillaris* in co-colture with *S. cerevisiae* on the chromatic characteristics of Montepulciano d'Abruzzo wine.

2. Materials and methods

2.1 Sampling site

Must samples *Vitis vinifera* cultivar (cv.) Montepulciano were kindly provided by a cellar located in Orsogna (Chieti, Abruzzo, Italy). Vineyards (42◦13' 01.5''N; 14◦14' 43.6''E), 403 m elevation, with calcareous, clayey soil received no irrigation, and were subjected to organic in accordance with Reg. EC 834/2007 (EC, 2007) since 2012. In particular, the pest management was achieved only through copper/sulphur-based products.

The *Vitis vinifera* cultivar (cv.) Montepulciano is the most important red variety of Abruzzo region, with over 35,000 ha of vineyards planted mainly along the Adriatic Coast. It is used for the production of high-quality red wines like Montepulciano d'Abruzzo Colline Teramane, and Terre Tollesi (or Tullum) wines which gained the DOCG (Designation of Controlled and Guaranteed Origin) recognition.

2.2 Strains origin

Ten strains of *St. bacillaris* (SB1, SB3, SB5, SB7, SB8, SB9, SB10, FUC9, FUC16, and FUC17) and a strain of *S. cerevisiae* (SRS1) were used in this study. All strains belong to the Culture Collection of the Microbial Biotechnology Laboratory (Department of BioScience and Technology for Food, Agriculture, and Environment – University of Teramo, Italy) and were previously characterized (Suzzi et al., 2012; Tofalo et al., 2016; Perpetuini et al., 2021). All strains were isolated from Montepulciano d'Abruzzo grapes, with the only exception of FUC9, FUC16, and FUC17 which were isolated from Nero Antico di Pretalucente grapes. The strains were cultivated under aerobic conditions at 28°C for 48 hours on YPD medium, which consists of 1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose, as per standard practice. The strains were preserved at a temperature

129 of -80°C in YPD broth supplemented with glycerol (Sigma-Aldrich, Milan, Italy) at a final
130 concentration of 20% v/v.
131
132 *2.3 Small scale vinification*
133 Must from Montepulciano grapes was obtained after 3 days of cryomaceration at 4 °C in contact with
134 skins and seeds, was divided in aliquots of 400 mL, and pasteurized (Caridi, Sidari, Kraková, Kuchta,
135 & Pangallo, 2015). Fermentations were carried out in 500 mL Erlenmeyer flasks closed with a Müller
136 valve filled with sulfuric acid. Each flask contained 400 mL of the must obtained as described above
137 (248 g/L – 24.6 °Bx of fermentable sugars, 7.67 titratable acidity, pH of 3.4). The fermentation was
138 carried out under static conditions at 25°C. The flasks were inoculated with pre-cultures grown in the
139 same must for 48 h. Strains were co-inoculated at a final concentration of 6 Log CFU/mL. The cell
140 concentration was determined by counting under light microscopy. *Starmerella bacillaris* strains
141 were inoculated both as planktonic and biofilm-detached cells. Biofilm-detached cells were prepared
142 as previously described (Perpetuini et al., 2022). Briefly, biofilms were formed inoculating cells in
143 flat-bottom 6-well cell culture plates (Costar, Corning, NY, USA). After 7 days sessile cells were
144 detached using a sterile cell scraper (Perpetuini et al., 2022). These cells are referred as biofilm
145 detached cells and used for further experiments.
146 The kinetics of fermentation were assessed on a daily basis through the observation of weight
147 reduction resulting from the emission of CO2. Once a stable weight was reached, the fermentation
148 process was considered as ended. Three biological and three technical replicates were conducted.
149
150 *2.4 Viable yeasts count*
151 Serial dilutions were prepared in physiological solutions (NaCl 0.85% w/v). Cell suspensions were
152 plated on WLN agar, which allows the visual differentiation of *St. bacillaris* and *S. cerevisiae* yeast
153 species. Plates were incubated at 28 °C for 3–5 days before counting. In this medium, *St. bacillaris*
154 forms flat, light to intense green colonies, while *S. cerevisiae* forms creamy white colonies, with light

155 shades of green on the top facilitating the concurrent enumeration of both species during the
156 fermentation process. Plate count was performed after 7 days of alcoholic fermentation (T7) and at
157 the end of fermentation (Tf). All analyses were performed in triplicate.
158
159 *2.5 Main oenological parameters*
160 FOSS WineScan™ FT120 rapid scanning Fourier Transform Infrared Spectroscopy with FOSS
161 WineScan software version 2.2.1 was used to analyse the main physicochemical parameters.
162 Previously, the equipment was calibrated using wine samples tested according to established OIV
163 protocols (OIV, 2023). The pH was determined using a pH meter. Pyruvate, polyphenols, and
164 anthocyanins were determined enzymatically using commercial kits from Steroglass (Perugia, Italy)
165 according to the manufacturer's instructions. Acetaldehyde concentration was determined by gas
166 chromatography with a flame ionization detector (GC-FID) using Agilent Technologies 6850
167 equipment (Palo Alto, CA), according to Morata et al. (2015).
168
169 *2.6 Wine color analysis*
170 Wine color analysis was carried out using a colorimeter (Minolta, Chroma Meter CR-5). Clarity (L*),
171 red/green color component (a*), and blue/yellow color component (b*), and their derived magnitudes,
172 chroma (C*), and tone (h*), were determined using glass cuvettes with a path length of 0.2 cm after
173 clarification of the samples by centrifugation (OIV, 2023). The color of wines obtained with
174 planktonic cells and biofilm-detached cells was compared, and the color difference was expressed as
175 $\Delta E = [(\Delta L)2 + (\Delta a^*)2 + (\Delta b^*)2]$ ½ (Ayala, Echavarri, & Negueruela, 1997).
176
177 *2.7 Confocal laser scanning microscopy*
178 Biofilms were visualized through the utilization of confocal laser scanning microscopy (CLSM) with
179 the Nikon A1-R confocal imaging system, which was operated through the Nikon NIS-Elements
180 interface (Version 4.40, Nikon Corp., Tokyo, Japan). The analyses were conducted in triplicate

2.8 Statistical analysis

The ANOVA test was performed using XLStat 2014 software (Addinsoft, New York, NY, USA) and was applied on the oenological parameters, the content of polyphenols, anthocyanins, pyruvic acid, and acetaldehyde, and the chromatic characteristics of wine in order to identify the significant differences. The Bonferroni correction was applied. The Pearson's Correlation matrix analysis was performed using XLStat 2014 software considering the content of anthocyanins, polyphenols, glycerol, ethanol, pyruvic acid, acetaldehyde, the number of cells and the chromatic charactristics of wines.

Moreover, a machine learning (ML) framework to estimate the wine color from anthocyanins, polyphenols, the number of viable yeast cells, pyruvic acid, and acetaldehyde was developed. Particularly, a Support Vector Regressor (SVR) with a radial basis function kernel was used to estimate, independently, the L*, a*, and B features, using as input anthocyanins, polyphenols, the number of viable yeast cells, pyruvic acid, and acetaldehyde. The input features were normalized (z score), and the generalization performance of the model was tested employing a nested cross validation (nCV). The nCV approach involves partitioning the available data into distinct folds, and subsequently training the model in an iterative and nested manner on all folds except for one. The outer loop and inner loop serve distinct purposes in the model evaluation process. While the outer loop is responsible for estimating the model's performance across iterations, the inner loop is tasked with identifying the optimal hyperparameter through validation. In this study, a 5-fold CV was performed. The performance of the models was evaluated considering the correlation coefficient between the measured and predicted variables.

3. Results and discussion

The microbial metabolism is influenced by the lifestyle of microrganisms: sessile cells, as well as biofilm-detached cells, frequently express phenotypes that are different from their planktonic counterparts (Bastard et al., 2016; Pannella et al., 2020). Recent studies reported the ability of *St.*

207 *bacillaris* to form biofilms on different abiotic surfaces, revealing that sessile and planktonic cells
208 can influence the characteristics of wines in different ways (Perpetuini et al., 2021; 2022). In
209 particular, wines fermented with sessile cells allowed to obtain wines with higher concentrations of
210 esters and glycerol and with a different sensory profile. In order to better understand the contribution
211 of biofilm-detached cells to wine characteristics, in this study, the effect of biofilm-detached cells
212 and planktonic cells on the chromatic characteristics of Montepulciano d'Abruzzo wine was tested.
213
214 *3.1 Determination of biofilm forming ability*
215 The biofilms formed by *St. bacillaris* strains were visualized, for the first time, by CSLM. CSLM
216 analysis revealed that all strains were able to form biofilm, in a strain-dependent way. Fig. 1 showed
217 a three-dimensional reconstruction of *St. bacillaris* biofilms resulting from the compilation of a series
218 of individual xy sections taken across the z axis. The images showed a biofilm organized in a
219 monolayer of sessile cells surrounded by an extracellular polysaccharide-like substance. Although,
220 the biofilm did not cover the entire surface of the glass, the cells adhered, flattened, and produced
221 extracellular material that bonded them to the surface, after which they finally organized themselves
222 in microcolonies (Fig. 1).
223
224 *3.2 Oenological parameters and yeast viability*
225 The presence of *S. cerevisiae* allowed the fermentation process to end after 15 days. However, when
226 *St. bacillaris* was inoculated as biofilm-detached cells, a slower fermentative activity was observed
227 (Supplementary Figure 1). In fact, the trials inoculated with *S. cerevisiae* and biofilm-detached cells
228 of *St. bacillaris* showed a lower fermentative power, evaluated as CO_2 evolution (g/100 ml) after 2
229 days of fermentation. The CO_2 evolved in trials inoculated with *S. cerevisiae* and planktonic cells of
230 *St. bacillaris* ranged from 1.6 g CO2/100mL to 5.1 *g* CO2/100mL, while in trials inoculated with *S.*
231 *cerevisiae* and biofilm-detached cells of *St. bacillaris* from 0.88 g CO_2/100mL to 3.65 g CO_2/100 mL
232 after 2 days. This slower fermentation ability could be related to the metabolism of sessile cells or

233 biofilm-detached cells, which are characterized by a different metabolism, e.g., in terms of metabolite
234 production, than their planktonic counterparts (Bojsen et al., 2012). Probably, these differences could
235 slow down the fermentation process, influencing the interactions between *St. bacillaris* and *S.*
236 *cerevisiae* strains. Rossouw et al. (2015, 2018) showed that changes in adhesion properties of *S.*
237 *cerevisiae* significantly affected the survival of other yeast species. Probably, this evidence could be
238 true also for *St. bacillaris* strains used in this study. Moreover, the inoculation of biofilm-detached or
239 planktonic cells of *St. bacillaris* could cause a differential expression of S. cerevisiae genes involved
240 in the fermentation process (Pourcelot et al., 2023). It should be also noted that, the different yeast
241 species could have overlapping nutritional requirements leading to competition for nutrients such as
242 amino-acids or vitamins (Evers et al. 2021). Probably, biofilm-detached cells could be more
243 competitive with *S. cerevisiae* and steal nutrients from it during the first steps of alcoholic
244 fermentation. This observation is in agreement with previous studies which highlighted that different
245 couples of *St. bacillaris* and *S. cerevisiae* can influence the growth dynamics, the fermentation
246 behavior and, as a consequence, wine composition in a couple-dependent manner (Englezos et al.,
247 2019). On the basis of our results, the interaction between these 2 yeasts is not only couple-ependent,
248 but depends also on *St. bacillaris* lifestyle.
249 The lifestyle of *St. bacillaris* did not influence the main oenological parameters of Montepulciano
250 d'Abruzzo wines (Table 1). Significant differences were only observed for the content of ethanol and
251 glycerol. A slight reduction of ethanol was detected when *St. bacillaris* was inoculated as biofilm
252 detached cells. Probably, in biofilm-detached cells the acetaldehyde pathway is less active than in
253 planktonic ones. In fact, when biofilm-detached cells are inoculated a reduction of ethanol content
254 and an increase of glycerol concentration have been detected. Effectively, the low production of
255 ethanol is strictly linked to the low activity of the acetaldehyde pathway. It is already known that this
256 behaviour has large-scale effects on the metabolic fluxes, necessitating higher glycerol production to
257 compensate for reduced ethanol production and to maintain cells' redox balance (Ansell, Granath,
258 Hohmann, Thevelein, & Adler, 1997) (Fig. 2). As a direct consequence, increased production of

259 pyruvate and amino acids and larger amounts of alcohols derived from alanine, leucine, valine, and
260 isobutanol, as well as metabolites from glyceraldehyde-3-phosphate, are shown (Comitini et al.,
261 2021).
262 Glycerol is the most abundant yeast metabolism by-product after ethanol and CO_2. This is a non
263 volatile 3-hydroxy alcohol and appears to contribute to the mouthfeel and sweetness of wine in the
264 range of 5–12 g/L (Ivit, Longo, & Kemp, 2020). Wines obtained with biofilm-detached cells of *St.*
265 *bacillaris* were characterized by a higher content of glycerol than those obtained with planktonic
266 cells. In particular, wines produced with biofilm-detached cells produced wines with a content of
267 glycerol ranging from 6.06 g/L (SRS1+SB8) to 9.38 g/L (SRS1+SB9), while the planktonic ones
268 ranged from 5.03 g/L (SRS1+SB7) to 8.12 g/L (SRS1+FUC17) (Table 1). Similar results have already
269 been reported when *St. bacillaris* was adhered to oak chips (Perpetuini et al., 2021; 2023). The
270 glycerol biosynthetic genes are up-regulated in biofilms, and the amounts of glycerol are significantly
271 higher in sessile cells compared to planktonic cells (Desai et al., 2013). In fact, the decreased glycerol
272 levels result in the down-regulation of biofilm adhesin genes such as ALS1, ALS3, and HWP1 (Desai
273 et al., 2013). It is unclear why glycerol and biofilm formation should be so closely linked. However,
274 according to Desai et al. (2013) glycerol biosynthesis is essential for proper expression of numerous
275 biofilm regulated genes, including adhesin genes. The obtained results highlighted that the number
276 of *St. bacillaris* viable yeast cells in the fermentation trials performed with planktonic cells was
277 characterized by a stronger cell decay ($p < 0.05$) than that observed in trials fermented with biofilm
278 detached ones after 7 days of fermentation. In fact, a decrease of about 2 Log CFU/mL was observed:
279 the number of *St. bacillaris* viable cells in fermentation trials performed with planktonic cells showed
280 a mean value of about 2.42 log CFU/mL, while that of trials inoculated with biofilm-detached cells
281 was 3.91 log CFU/mL (Fig. 3). On the contrary, the number of *S. cerevisiae* viable cells was similar
282 in both conditions. At the end of alcoholic fermentation, the number of biofilm-detached *St. bacillaris*
283 cells was about 2 Log CFU/mL, while this yeast was not detected in the trials performed with
284 planktonic cells. *S. cerevisiae* cells were detected in similar concentration (Fig. 3). This finding could

285 be related to the ability of biofilm-detached cells to better face the stresses of alcoholic fermentation.
286 In fact, as reported by Guilhen et al. (2016), cells dispersed from biofilms have a high stress response
287 because they are transcriptionally closer to their parent cells in biofilm form than to cells in planktonic
288 form.
289
290 *3.3 Biofilm-detached cells increase the content of pyruvic acid, anthocyanin, and polyphenols*
291 Acetaldehyde is a potent volatile flavor compound that, at low levels, gives a pleasant fruity aroma,
292 but at high concentrations (higher than 100–125 mg/L), it possesses a pungent, irritating odor (Berg,
293 Filipello, Hinreiner, & Webb, 1955). Moreover, it plays a key role in the increase in color (Liu &
294 Pilone, 2000). However, it should be noted that the International Agency for Research on Cancer
295 (IARC) classified acetaldehyde as “possibly carcinogenic to humans (Group 2B)” and, in
296 combination with its oral intake via alcoholic beverages, as “carcinogenic to humans (Group 1)”.
297 According to the criteria set out in Regulation (EC) No 1272/2008 (Classification, Labeling and
298 Packaging regulation), acetaldehyde is classified as carcinogenicity category 1B (may cause cancer)
299 and germ cell mutagenicity category 2 (suspected of causing genetic defects) meeting the criteria to
300 be considered a carcinogenic, mutagenic, and/or toxic for reproduction (Cartus et al., 2023).
301 Acetaldehyde content was similar in both conditions; in fact, a mean value of 40 mg/L was detected
302 in wines obtained with planktonic and biofilm-detached cells.
303 The content of pyruvic acid was higher in wines obtained with biofilm-detached cells. In particular,
304 its content ranged from 45.99 mg/L (SRS1+SB10) to 48.19 mg/L (SRS1+FUC17) and from 41.13
305 mg/L (SRS1+SB9) to 45.9 mg/L (SRS1+FUC16) in wines fermented with biofilm-detached and
306 planktonic cells, respectively (Table 2). It seems that biofilm-detached cells are more efficient at
307 redirecting sugar consumption for the production of alternative compounds, rather than ethanol, than
308 planktonic ones. These alternative compounds could be glycerol and pyruvic acid produced via
309 glycerol-pyruvic metabolisms (Fig. 2). The production of pyruvic acid has already been described in
310 *St. bacillaris* (Magyar, Nyitrai-Sárdy, Leskó, Pomázi, & Kállay, 2014; Mangani, Buscioni, Collina

311 Bocci, & Vincenzini, 2011). Generally, the production of pyruvate by wine yeasts varies from 50
312 mg/L to 120 mg/L (Morata, Gómez-Cordovés, Colomo, & Suárez, 2003). Generally, the production
313 of pyruvate, a metabolic intermediate in the biosynthesis of acetyl CoA, grows at the beginning of
314 fermentation, while its concentration decreases at the end of alcoholic fermentation. As the
315 fermentation process progresses and the availability of nutrients decreases, yeasts utilize the pyruvate
316 that was previously secreted during the earlier stages of fermentation (Morata et al., 2003).
317 The production of pyruvic acid is essential to improving wine color. In fact, according to Morata et
318 al. (2003), a linear relationship between vitisin A production and pyruvate levels can be observed.
319 Therefore, the use of biofilm-detached cells, characterized by a higher production of pyruvic acid
320 than planktonic ones, could be an interesting strategy to modulate wine color. This may be especially
321 important for red wines destined to be aged (especially if they are aged in the barrel) or are to undergo
322 a second fermentation (e.g., sparkling wines). The color of wine is also influenced by anthocyanins
323 and polyphenols, as well as the extraction, absorption and preservation phenomena of anthocyanins.
324 Therefore, their content was also evaluated. The anthocyanins were mainly absorbed by planktonic
325 cells; in fact, wines obtained with biofilm-detached cells showed levels of anthocyanins ranging from
326 506.8 mg/L (SRS1+FUC16) to 659.9 mg/L (SRS1+SB7), while those fermented with free cells of *St.*
327 *bacillaris* ranged from 518.8 mg/L (SRS1+FUC9) to 612.6 mg/L (SRS1+SB1) (Table 2). Similarly,
328 the content of polyphenols was higher in wines inoculated with biofilm-detached cells. In fact, the
329 content of polyphenols ranged from 5.7 g/L gallic acid equivalents to 6.9 g/L gallic acid equivalents,
330 and from 5 g/L gallic acid equivalents to 5.7 g/L gallic acid equivalents in biofilm-detached and
331 planktonic cells, respectively.
332 The concentration of polyphenols in wines is influenced by viticulture (grape variety and clone, light
333 exposure, degree of ripeness), yeast strains, and vinification process (destemming, crushing, pre
334 fermentation maceration, alcoholic fermentation, pressing) (Jagatic Korenika, Tomaz, Preiner,
335 Plichta, & Jeromel, 2021). For instance, according to Lisov et al. (2020) the extraction of phenolic

336 compounds during alcoholic fermentation is affected by maceration time. The best results were
337 obtained after 15 days of maceration, with exceptions of gallic acid, catechin, and myricetin.
338 Regardless of the adhesion properties of *St. bacillaris*, a negative relationship can be established
339 between the number of viable yeast and the content of anthocyanins and polyphenols, suggesting that
340 their release or adsorption is mainly dependent on the vitality of yeasts. Probably, the differences
341 observed in this study could be related to the viability of the yeast cells. In fact, cells embedded in a
342 biofilm, as well as biofilm-detached cells, are more resistant to stresses than planktonic ones.
343 According to Echeverrigaray, Scariot, Menegotto, and Delamare (2020), a negative correlation
344 between pigment adsorption and both cell viability and cell wall/membrane integrity can be observed.
345 Irrespective of their adsorptive potential during the process of wine fermentation, viable cells
346 demonstrated a limited ability to adsorb anthocyanins. Conversely, permeabilized yeast cells
347 exhibited a high capacity for pigment adsorption.
348
349 *3.4 Oenological parameters and wine color*
350 The color of red wine is a major concern for the wine industry since it strongly affects consumer
351 demands. Anthocyanin content is the main reason for the color of red wine and depends on the grape
352 variety, degree of grape ripeness, soil, and climatic conditions. It undergoes a progressive change
353 from production to consumption of any wine due to polymerization, copigmentation, and oxidation
354 reactions. Therefore, it is important to evaluate the effect of the different oenological parameters on
355 wine color and try to predict it on the basis of these parameters.
356 Concerning the chromatic characteristics of wine, b* values (blue/yellow color) were all low,
357 reflecting the low presence of yellow color component in Montepulciano d'Abruzzo wines (Table 3).
358 Wines obtained with biofilm-detached cells of *St. bacillaris* had lower values of b* and h* than those
359 obtained with planktonic cells. The lower value of h* leads to purple or ruby red, while higher values
360 lead to brick red or reddish brown. These wines also showed higher a* values, indicating the presence
361 of a stronger red color than the others, and lower clarity (L*) (Table 3). No significant differences

362 were obtained for the parameter c*, which represents the psychometric chroma. It is important to
363 underline that in 6 trials out of 10, the E values were higher than 3 CIELAB units (Table 4), indicating
364 that the color differences between wines obtained from planktonic and biofilm-detached cells could
365 be perceived by human eyes (Martinez, Melgosa, Perez, Hita, & Negueruela, 2001). These results
366 suggested that yeast's absorption of phenolic compounds could result in an increase in yellow color
367 and a reduction of blue and red nuances, indicating that not only the choice of yeast strains but also
368 their lifestyle (planktonic vs. biofilm-detached) is important to defining the color of wine. The content
369 of anthocyanins could help explain these differences. In fact, the content of anthocyanins is negatively
370 correlated with L* values, suggesting their significant contribution to color intensity (i.e., a smaller
371 L* value), and positively with a*, indicating their contribution to red wine color.
372 A correlation matrix was constructed to establish the relationship between the variables considered.
373 In biofilm-detached cells, anthocyanin content was positively correlated with the concentration of
374 polyphenols, the number of cells, and a* values. Positive relations were also present between
375 polyphenols, a* values, and the number of cells. a* values were positively correlated with the number
376 of cells and the content of acetaldehyde (Table 5). In planktonic cells, anthocyanins were positively
377 correlated with the concentration of polyphenols and the number of cells. Polyphenols were positively
378 related to the number of cells and a* values and negatively to pyruvic acid. a* values correlated
379 positively with the number of cells and negatively with the amount of pyruvic acid. As expected, the
380 content of polyphenols and anthocyanins is essential to improve red wine color in both conditions.
381 Moreover, the number of viable cells is another key factor for the determination of red wine color. In
382 fact, viable cells show a limited ability to adsorb anthocyanins on their cell wall (Echeverrigaray et
383 al., 2020).
384 A regression model was developed to predict the color of wine based on the following parameters:
385 anthocyanins, polyphenols, the number of viable yeasts, pyruvic acid, and acetaldehyde. The
386 developed model behaved fairly well for the prediction of L*, a*, and b* when biofilm detached cells
387 are inoculated. In fact, r2 was 0.727, 0.878, and 0.628 for L*, a*, and b*, respectively. Good regression

388 coefficients were observed also for planktonic cells: r2 values of 0.628, 0.748, and 0.623 for L*, a*,
389 and b*, respectively (Fig. 4). The regression coefficient of determination of cross-validation showed
390 that the analysis of wine samples with these methods could allow predictions of wine color. It should
391 be noted that the regression model behaved better in the presence of biofilm-detached cells,
392 suggesting that their use in winemaking could be useful to predict the color of wine more accurately.
393 However, to increase the accuracy and robustness of these prediction models and to employ them in
394 commercial applications, larger sample sets can be used in future studies.
395
396 **4. Conclusion**
397 The results obtained in this study offer first evidence of the role of *St. bacillaris* grown as biofim
398 detached cells in the determination of Montepulciano d'Abruzzo wine color. In particular, the co
399 inoculation of biofilm-detached cells of *St. bacillaris* and *S. cerevisiae* resulted in an increase of
400 glycerol, pyruvic acid, polyphenols and anthocyanins and a decrease of ethanol content. Moreover,
401 wines obtained with biofilm-detached cells had lower values of b* and h* and higher a* values,
402 indicating the presence of a stronger red color. Moreover, it should possible to predict the color of
403 young wines from must measurements. The developed model behaved fairly well for the prediction
404 of L*, a*, and b* when biofilm detached cells were inoculated. This approach provides an important
405 starting point for further identification and prediction of wine quality factors from these parameters.
406 This kind of studies are of great importance to help the oenologists to better manage wine polyphenols
407 through the correct choice of yeast strain or inoculum strategy.
408
409 **CRediT authorship contribution statement** Rosanna Tofalo: conceptualization, supervision,
410 funding acquisition, Writing – review & editing. Luca Valbonetti: CLSM analysis. Rossana Sidari:
411 investigation. Alessio Pio Rossetti: investigation, formal analysis. Giorgia Perpetuini: formal
412 analysis, data curation, Writing – original draft, Writing – review & editing. Carlo Perla: Writing –
413 review & editing, Camillo Zulli: Writing – review & editing

Declaration of Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability Data will be made available on request.

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429 **Figure captions**

430 Figure 1. CLSM images of *St. bacillaris*. (A) ×100 3D images of strains. (B) ×100 3D images from

431 the frontal view of strains.

432

433 Figure 2. Carbon metabolism in yeasts. ADH: alcohol dehydrogenase; GPDH: glycerol-3-phosphate

434 dehydrogenase; G3P: glycerol-3-phophatase; PDC: pyruvate decarboxylase; DHAP:

435 dihydroxyacetone phosphate; GA3P: glyceraldehyde-3-phosphate.

436

437 Figure 3. Box plot showing the number of viable yeasts after 7 days (T7) and at the end of alcoholic

438 fermentation (Tf). PL: planktonic, BD: biofilm-detached. ns: $p>0.05$, $*p<0.05$

439

440 Figure 4. Correlation between obtained L*, a* and b* values and predicted ones.

441

442 Supplementary Figure 1. Fermentation kinetics. P: planktonic, BD: biofilm-detached

445 Table 1 Main oenological parameters obtained at the end of alcoholic fermentation using co-cultures of *S. cerevisiae* and *St. bacillaris* grown as

446 planktonic or sessile cells. Different letters in the same line indicates significant differences ($p<0.05$)

447

Trial	Alcohol (% v/v)		Residual sugars (g/L)		pH		Titratable acidity (g/L)*		Volatile acidity (g/L)**		Glycerol	
	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic
SRS1+SB1	13.92±0.32^{A}	14.12±0.32^{A}	0.57±0.03^{A}	0.55±0.03^{A}	3.33±0.13^{A}	3.31±0.05^{A}	5.39±0.33^{A}	5.37±0.43^{A}	0.52±0.03^{A}	0.53±0.08^{A}	7.54±0.23^{B}	5.36±0.44^{A}
SRS1+SB3	13.74±0.83^{A}	14.24±0.53^{A}	0.34±0.08^{A}	0.36±0.03^{A}	3.3±0.27^{A}	3.32±0.17^{A}	6.44±0.37^{A}	6.43±0.84^{A}	0.45±0.03^{A}	0.48±0.03^{A}	7.97±0.44^{B}	5.27±0.35^{A}
SRS1+SB5	14.25±0.54^{A}	14.15±0.99^{A}	0.36±0.03^{A}	0.31±0.02^{A}	3.33±0.08^{A}	3.35±0.14^{A}	6.29±0.12^{A}	6.21±0.32^{A}	0.45±0.08^{A}	0.48±0.04^{A}	8.89±0.43^{B}	6.89±0.93^{A}
SRS1+SB7	13.71±0.78^{A}	13.93±0.13^{A}	0.59±0.04^{A}	0.51±0.04^{A}	3.33±0.15^{A}	3.34±0.34^{A}	6.66±0.93^{A}	6.73±0.34^{A}	0.49±0.07^{A}	0.51±0.09^{A}	6.14±0.22^{B}	5.03±0.56^{A}
SRS1+SB8	13.73±0.23^{A}	14.16±0.23^{A}	0.33±0.06^{A}	0.31±0.07^{A}	3.32±0.07^{A}	3.31±0.14^{A}	6.67±0.23^{A}	6.65±0.98^{A}	0.48±0.03^{A}	0.49±0.02^{A}	6.06±0.89^{B}	5.33±0.29^{A}
SRS1+SB9	13.82±0.67^{A}	14.18±0.43^{A}	0.36±0.06^{A}	0.31±0.03^{A}	3.34±0.16^{A}	3.33±0.04^{A}	6.43±0.32^{A}	6.3±0.67^{A}	0.58±0.02^{A}	0.57±0.06^{A}	9.38±0.77^{B}	8.1±0.93^{A}
SRS1+SB10	13.77±0.37^{A}	14.23±0.57^{A}	0.24±0.03^{A}	0.22±0.02^{A}	3.31±0.21^{A}	3.33±0.17^{A}	6.7±0.53^{A}	6.43±0.75^{A}	0.56±0.13^{A}	0.58±0.11^{A}	9.16±0.98^{B}	6.87±0.37^{A}
SRS1+FUC9	13.81±0.92^{A}	14.16±0.65^{A}	0.31±0.05^{A}	0.32±0.04^{A}	3.3±0.05^{A}	3.3±0.07^{A}	6.12±0.32^{A}	5.97±0.09^{A}	0.41±0.05^{A}	0.43±0.07^{A}	9.08±0.32^{B}	7.18±0.36^{A}
SRS1+FUC16	13.92±0.12^{A}	14.25±0.22^{A}	0.33±0.06^{A}	0.34±0.03^{A}	3.29±0.05^{A}	3.31±0.08^{A}	5.61±0.98^{A}	5.65±0.73^{A}	0.46±0.08^{A}	0.48±0.03^{A}	9.13±0.66^{B}	7.17±0.32^{A}
SRS1+FUC17	13.51±0.43^{A}	14.33±0.84^{B}	0.39±0.05^{A}	0.38±0.02^{A}	3.28±0.03^{A}	3.31±0.13^{A}	5.78±0.32^{A}	5.72±0.12^{A}	0.45±0.03^{A}	0.42±0.05^{A}	9.19±0.43^{B}	8.12±0.76^{A}

448 * Expressed as tartaric acid.

449 ** Expressed as acetic acid.

450

451

452 Table 2 Anthocyanins, and polyphenols content at the end of alcoholic ferm entation using co-cultures of *S. cerevisiae* and *St. bacillaris* grown as

453 planktonic or sessile cells. Different letters in the same line indicates significant differences ($p<0.05$)

454

Strain	Pyruvic acid (mg/L)		Anthocyanins (mg/L)		Polyphenols (g/L)		Acetaldehyde (mg/L)	
	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic
SRS1+SB1	46.96±6.98^{B}	41.46±6.77^{A}	616.42±56.91^{A}	612.61±45.19^{A}	6.93±2.81^{B}	5.12±0.42^{A}	39.32±12.76^{A}	40.22±13.78^{B}
SRS1+SB3	47.23±12.54^{B}	42.63±5.92^{A}	604.83±43.13^{A}	599.53±67.31^{A}	6.34±0.62^{A}	5.75±0.33^{A}	32.13±9.54^{A}	32.59±9.54^{A}
SRS1+SB5	47.87±11.65^{B}	43.81±11.41^{A}	570.61±57.94^{B}	560.25±53.15^{A}	5.72±1.33^{A}	5.36±0.55^{A}	30.38±3.87^{A}	30.16±8.33^{A}
SRS1+SB7	47.09±9.54^{B}	41.77±13.76^{A}	659.95±28.15^{B}	539.76±65.93^{A}	6.84±0.41^{A}	5.22±1.37^{A}	43.77±12.77^{B}	45.34±10.65^{A}
SRS1+SB8	46.13±5.99^{B}	42.66±10.54^{A}	588.32±92.72^{B}	537.91±89.41^{A}	5.95±1.75^{A}	5.14±0.69^{A}	50.45±11.23^{A}	49.41±9.45^{A}
SRS1+SB9	47.55±15.61^{B}	41.13±6.98^{A}	632.74±72.56^{B}	523.94±36.12^{A}	6.76±1.26^{B}	5.26±0.83^{A}	38.67±6.45^{A}	38.56±14.34^{A}
SRS1+SB10	45.99±8.43^{B}	43.68±12.81^{A}	643.81±55.91^{B}	544.62±33.62^{A}	6.93±0.54^{B}	5.32±0.55^{A}	41.56±9.87^{A}	41.76±9.65^{A}
SRS1+FUC9	46.08±7.23^{B}	44.80±13.86^{A}	593.74±69.23^{B}	518.14±98.13^{A}	5.95±1.27^{A}	5.15±1.13^{A}	37.98±12.65^{A}	39.54±7.33^{B}
SRS1+FUC16	47.54±5.72^{B}	45.91±9.66^{A}	506.86±73.85^{A}	583.86±88.35^{B}	5.72±0.93^{A}	5.23±0.55^{A}	41.33±8.65^{A}	41.98±18.45^{A}
SRS1+FUC17	48.19±13.66^{B}	44.43±4.88^{A}	581.11±95.43^{A}	608.17±93.43^{B}	5.86±1.55^{A}	5.22±1.12^{A}	45.65±13.77^{B}	44.12±13.66^{A}

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456

457

459 Table 3 Main chromatic characteristics of obtained wines. Different letters in the same line indicates significant differences ($p<0.05$)

460

Trial	L*		a*		b*		C*		h*	
	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic	Biofilm-detached	Planktonic
SRS1+SB1	34.51±12.78^{A}	36.92±9.54^{A}	44.74±9.54^{B}	43.01±9.32^{A}	5.21±0.34^{A}	6.27±0.53^{A}	43.58±16.88^{A}	43.01±16.75^{A}	6.46±0.76^{A}	6.64±0.56^{A}
SRS1+SB3	36.84±15.43^{A}	36.97±12.43^{A}	43.45±11.65^{A}	43.13±16.98^{A}	5.38±0.53^{A}	5.8±1.09^{A}	43.65±9.43^{A}	43.35±14.85^{A}	6.31±1.16^{A}	6.42±1.27^{A}
SRS1+SB5	34.08±12.99^{A}	37.21±6.54^{B}	42.78±16.87^{A}	42.75±12.54^{A}	5.91±1.12^{A}	5.99±0.37^{A}	43.68±14.67^{A}	43.5±12.37^{A}	6.48±0.59^{A}	6.59±0.54^{A}
SRS1+SB7	34.34±13.87^{A}	37.15±19.54^{B}	44.66±12.34^{B}	42.78±19.22^{A}	5.71±0.76^{A}	5.85±1.45^{A}	42.88±16.43^{B}	41.97±11.84^{A}	6.51±0.27^{A}	6.98±1.24^{A}
SRS1+SB8	35.61±17.54^{A}	37.99±12.66^{A}	43.97±16.23^{B}	42.03±16.16^{A}	5.53±1.11^{A}	5.68±0.65^{A}	42.71±13.99^{A}	42.27±9.86^{A}	6.15±0.39^{A}	6.29±1.18^{A}
SRS1+SB9	36.87±11.43^{A}	37.11±13.18^{A}	43.5±17.93^{A}	42.84±8.44^{A}	5.2±0.85^{A}	5.92±1.12^{A}	43.12±11.59^{A}	42.71±11.43^{A}	5.54±0.51^{A}	6.67±0.54^{B}
SRS1+SB10	35.22±16.88^{A}	37.22±15.32^{B}	43.66±12.66^{B}	42.29±12.66^{A}	5.86±1.77^{A}	5.96±0.87^{A}	43.95±16.32^{A}	43.45±16.58^{A}	6.53±1.06^{A}	6.75±1.49^{A}
SRS1+FUC9	34.78±13.75^{A}	37.71±12.98^{B}	42.91±11.28^{B}	42.11±16.92^{A}	5.49±0.34^{A}	5.81±1.66^{A}	43.52±7.48^{A}	43.14±9.38^{A}	5.97±0.48^{A}	6.35±0.75^{A}
SRS1+FUC16	37.09±12.99^{A}	37.34±14.31^{A}	42.67±9.99^{B}	42.22±16.32^{A}	5.54±1.49^{A}	5.35±0.59^{A}	42.51±8.59^{A}	42.41±14.44^{A}	5.81±1.15^{A}	6.14±0.98^{A}
SRS1+FUC17	35.31±12.43^{A}	36.98±17.77^{B}	43.98±16.43^{B}	42.18±11.05^{A}	5.56±0.55^{A}	5.49±1.15^{A}	43.99±12.49^{B}	42.98±17.51^{A}	5.88±0.59^{A}	6.37±1.16^{B}

461

462

463 Table 4 Colour difference in CIELAB units (ΔE) between the wines derived from the inoculation of

464 *S. cerevisiae* and *St. bacillaris* grown as planktonic and biofilm-detached cells

465

Trial	ΔE
SRS1+SB1	3.15
SRS1+SB3	0.54
SRS1+SB5	3.13
SRS1+SB7	3.38
SRS1+SB8	3.07
SRS1+SB9	1
SRS1+SB10	2.43
SRS1+FUC9	3.05
SRS1+FUC16	0.57
SRS1+FUC17	4.09

466

467

468 Table 5. Correlation matrix for samples obtained with biofilm-detached (A) and planktonic (B) cells

469 A

	Anthocyanins	Polyphenols	Glycerol	Ethanol	Cells	Pyruvic acid	Acetaldehyde	L*	a*	b*
Anthocyanins	1	0.820	-0.307	-0.324	0.903	-0.353	0.058	-0.163	0.644	0.070
Polyphenols	0.820	1	-0.460	-0.197	0.832	-0.228	0.142	0.119	0.781	0.117
Glycerol	-0.307	-0.460	1	0.169	-0.314	0.336	-0.441	-0.368	-0.647	-0.148
Ethanol	-0.324	-0.197	0.169	1	-0.469	0.162	-0.646	-0.087	-0.456	0.396
Cells	0.903	0.832	-0.314	-0.469	1	-0.476	0.094	0.045	0.624	-0.009
Pyruvic acid	-0.353	-0.228	0.336	0.162	-0.476	1	-0.295	-0.277	-0.119	-0.132
Acetaldehyde	0.058	0.142	-0.441	-0.646	0.094	-0.295	1	0.215	0.483	-0.091
L*	-0.163	0.119	-0.368	-0.087	0.045	-0.277	0.215	1	0.014	-0.614
a*	0.644	0.781	-0.647	-0.456	0.624	-0.119	0.483	0.014	1	0.208
b*	0.070	0.117	-0.148	0.396	-0.009	-0.132	-0.091	-0.614	0.208	1

470

471

472

473

474 B

	Anthocyanins	Polyphenols	Glycerol	Ethanol	Cells	Pyruvic acid	Acetaldehyde	L*	a*	b*
Anthocyanins	1	0.679	-0.102	0.523	0.726	0.102	-0.180	0.203	0.441	-0.297
Polyphenols	0.679	1	-0.476	0.079	0.701	-0.503	-0.341	-0.210	0.657	0.435
Glycerol	-0.102	-0.476	1	0.269	-0.232	0.389	-0.058	0.504	-0.348	-0.500
Ethanol	0.523	0.079	0.269	1	0.035	0.528	-0.131	0.523	-0.175	-0.492
Cells	0.726	0.701	-0.232	0.035	1	-0.215	-0.132	0.048	0.555	0.170
Pyruvic acid	0.102	-0.503	0.389	0.528	-0.215	1	-0.012	0.491	-0.656	-0.661
Acetaldehyde	-0.180	-0.341	-0.058	-0.131	-0.132	-0.012	1	0.327	-0.382	-0.406
L*	0.203	-0.210	0.504	0.523	0.048	0.491	0.327	1	-0.534	-0.618
a*	0.441	0.657	-0.348	-0.175	0.555	-0.656	-0.382	-0.534	1	0.392
b*	-0.297	0.435	-0.500	-0.492	0.170	-0.661	-0.406	-0.618	0.392	1

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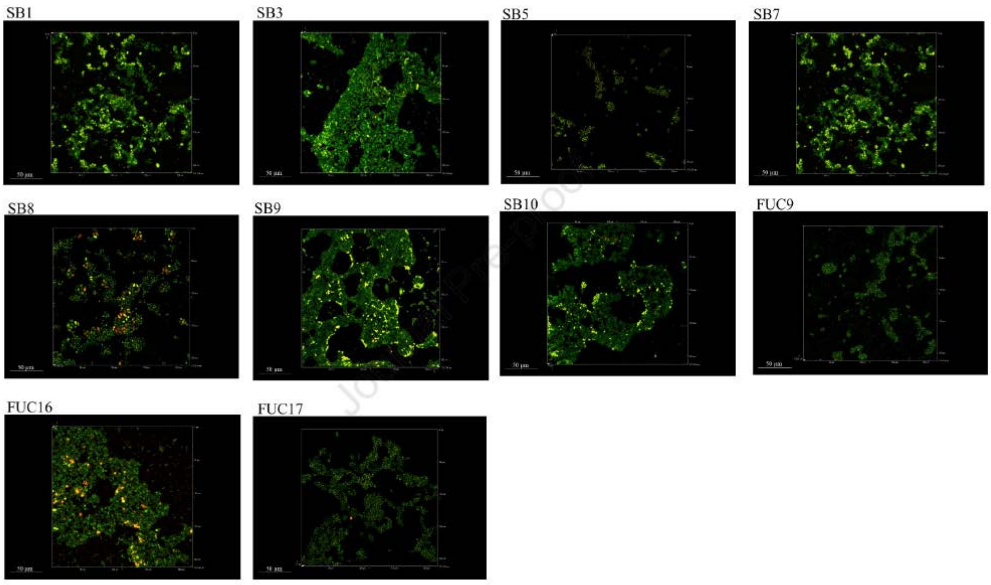


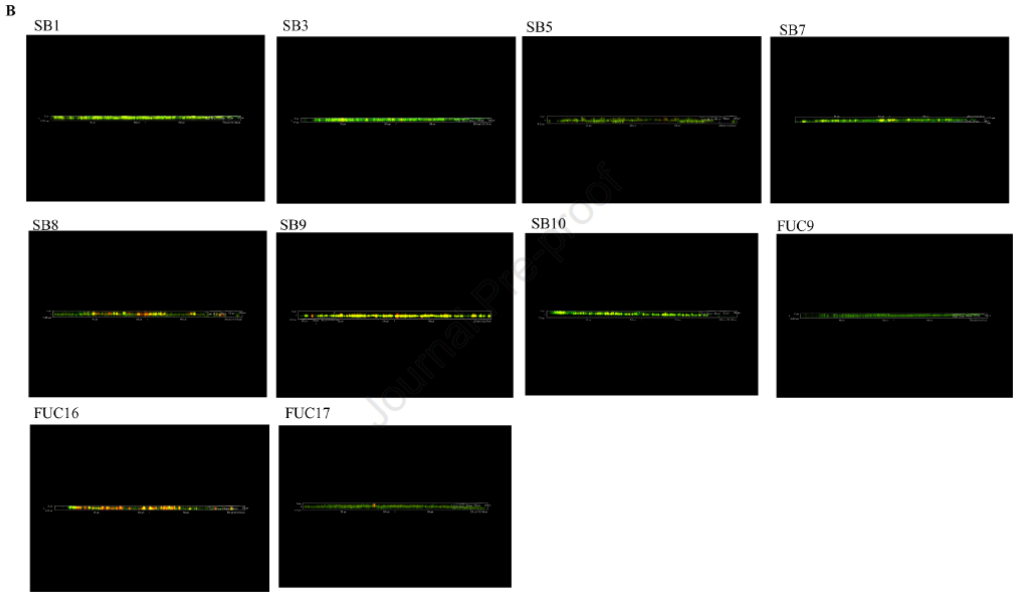
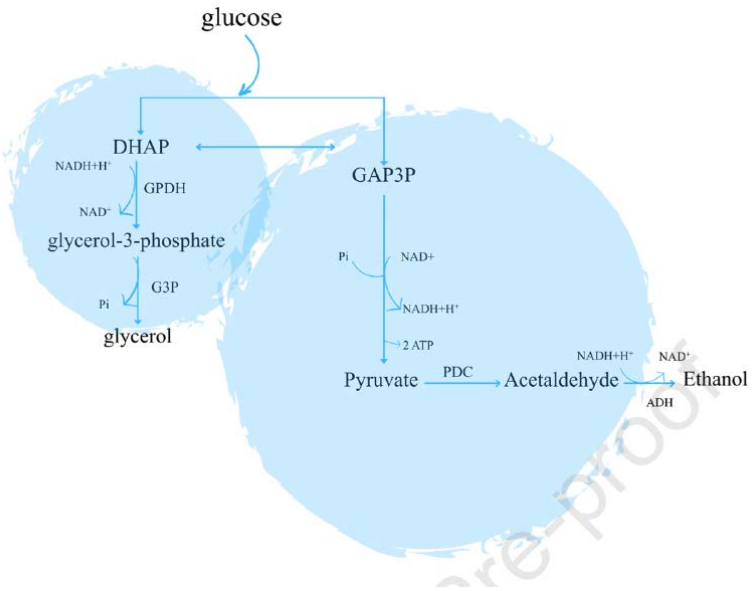


Figure 1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GPDH
NAD+
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Pi
glycerol
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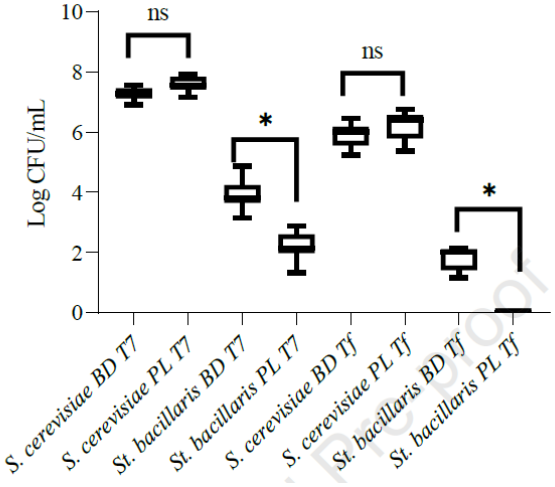
2 ATP
Pyruvate
PDC
Acetaldehyde
NADH+H+
NAD+
Ethanol
ADH

Figure 2.


Log CFU/mL
10
8
6
4
2
0
ns
*
ns
*
S. cerevisiae BD T7
S. cerevisiae PL T7
St. bacillaris BD T7
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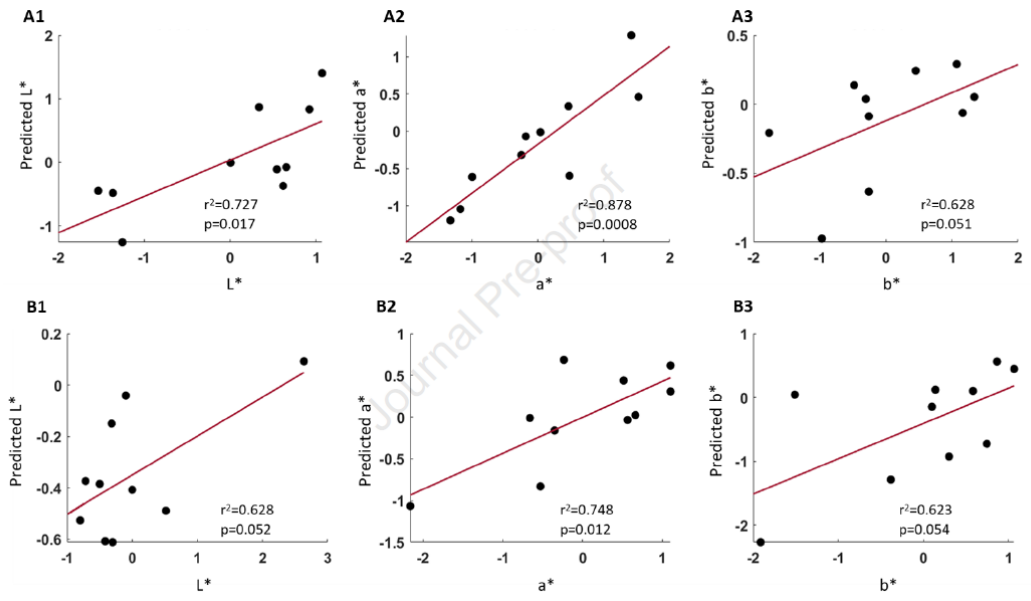
S. cerevisiae BD Tf
S. cerevisiae PL Tf
St. bacillaris BD Tf
St. bacillaris PL Tf

Figure 3.


A1
Predicted L*
L*
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p=0.017
A2
Predicted a*
a*
r²=0.878
p=0.0008
A3
Predicted b*
b*
r²=0.628
p=0.051
B1
Predicted L*
L*
r²=0.628
p=0.052
B2
Predicted a*
a*
r²=0.748
p=0.012
B3
Predicted b*
b*
r²=0.623
p=0.054

Figure 4.